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Comparison between Prototype Hybrid Capture 3 and Hybrid Capture 2 Human Papillomavirus DNA Assays for Detection of High-Grade Cervical Intraepithelial Neoplasia and Cancer

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We compared the performance of a prototype version of the Hybrid Capture 3 (HC3) human papillomavirus (HPV) DNA assay to the current generation Hybrid Capture 2 (HC2) assay, both of which target 13 oncogenic HPV types, for the detection of cervical intraepithelial neoplasia grade 3 and cancer (CIN3+) with cervicovaginal lavage specimens collected at enrollment into a 10-year cohort study at Kaiser Permanente (Portland, Oreg.). HC3 results for a risk-stratified sample ($n = 4,364$) were compared to HC2 results for the entire cohort ($n = 20,810$) with receiver operating characteristics curves, and the optimal cut points for both tests (relative light units [RLU]/positive control [PC]) for the detection of CIN3+ were determined. Specimens were also tested for HPV16 and HPV18 with separate HC3 type-specific probes. The optimal cut point for detecting CIN3+ was 1.0 RLU/PC for HC2, as previously shown, and was 0.6 RLU/PC for HC3. At the optimal cut points, HC3 and HC2 had similar screening performance characteristics for CIN3+ diagnosed at the enrollment visit. In analyses that included cases CIN3+ at enrollment and those diagnosed during early follow-up, HC3 had nonsignificantly higher sensitivity and equal specificity for the detection of CIN3+ compared to HC2; this increase in sensitivity was primarily the result of increased detection of CIN3+ in women who were 30 years of age or older and were cytologically negative ($P = 0.006$). We also compared the performance of the hybrid capture tests to MY09/11 L1 consensus primer PCR results ($n = 1,247$). HC3 was less likely than HC2 to test positive for specimens that tested positive by PCR for any untargeted types ($P < 0.001$). HC3 was less likely than HC2 to test positive for untargeted PCR-detected single infections with HPV53 ($P = 0.001$) and HPV66 ($P = 0.01$). There was good agreement between test positivity by PCR and by single type-specific HC3 probes for HPV16 (kappa = 0.76; 95% confidence interval [CI] = 0.71 to 0.82) and for HPV18 (kappa = 0.73; 95% CI = 0.68 to 0.79). In conclusion, we suggest that HC3 (≥0.6 RLU/PC) may be slightly more sensitive than and equally specific test as HC2 (≥1.0 RLU/PC) for the detection of CIN3+ over the duration of typical screening intervals.

Hybrid Capture 3 (HC3) (Digene Corporation, Gaithersburg, Md.) is being evaluated as the next generation of hybrid capture clinical assays that target 13 oncogenic HPV types for the detection of cervical precancerous cervical intraepithelial neoplasia grade 3 (CIN3) (11) and cancer (1, 9, 18). Hybrid Capture 2 (HC2), the predecessor of HC3 and an assay currently in clinical use, has been shown to have similar analytic sensitivity to some PCR methods for HPV DNA detection (2, 4, 10). HC2 is a sensitive test for the detection of CIN3 and cervical cancer (CIN3+) (13) and has received Food and Drug Administration approval for guiding the management of women with equivocal cytology (atypical squamous cells) (16, 19).

HC3, like the previous hybrid capture tests, relies on the formation of target HPV DNA-RNA probe heteroduplexes during the hybridization step in specimens containing sufficient HPV DNA and the chemiluminescent detection of these hybrids by with an alkaline phosphatase-conjugated monoclonal antibody specific to DNA-RNA complexes with dioxetane substrate in a 96-well enzyme-linked immunosorbent assay (ELISA) format (8). A primary technical distinction between HC3 and HC2 is that HC3 employs a biotinylated DNA oligonucleotide specific for selected HPV DNA sequences for the capture of the DNA-RNA complexes on streptavidin-coated wells, whereas HC2 uses wells coated with polyclonal antibody against DNA-RNA complexes for hybrid capture. The use of capture oligonucleotide instead of an immobilized antibody also diminishes the possibility of nonspecific RNA-DNA hybrids, present as the result of improperly alkali-denatured specimens, from binding to the microplate well and consequently may reduce false positivity for HC3 compared to HC2 (3, 10, 17).

To evaluate the performance of a prototype HC3 test for the detection of CIN3+ with probes that target the same set of 13 carcinogenic HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56,

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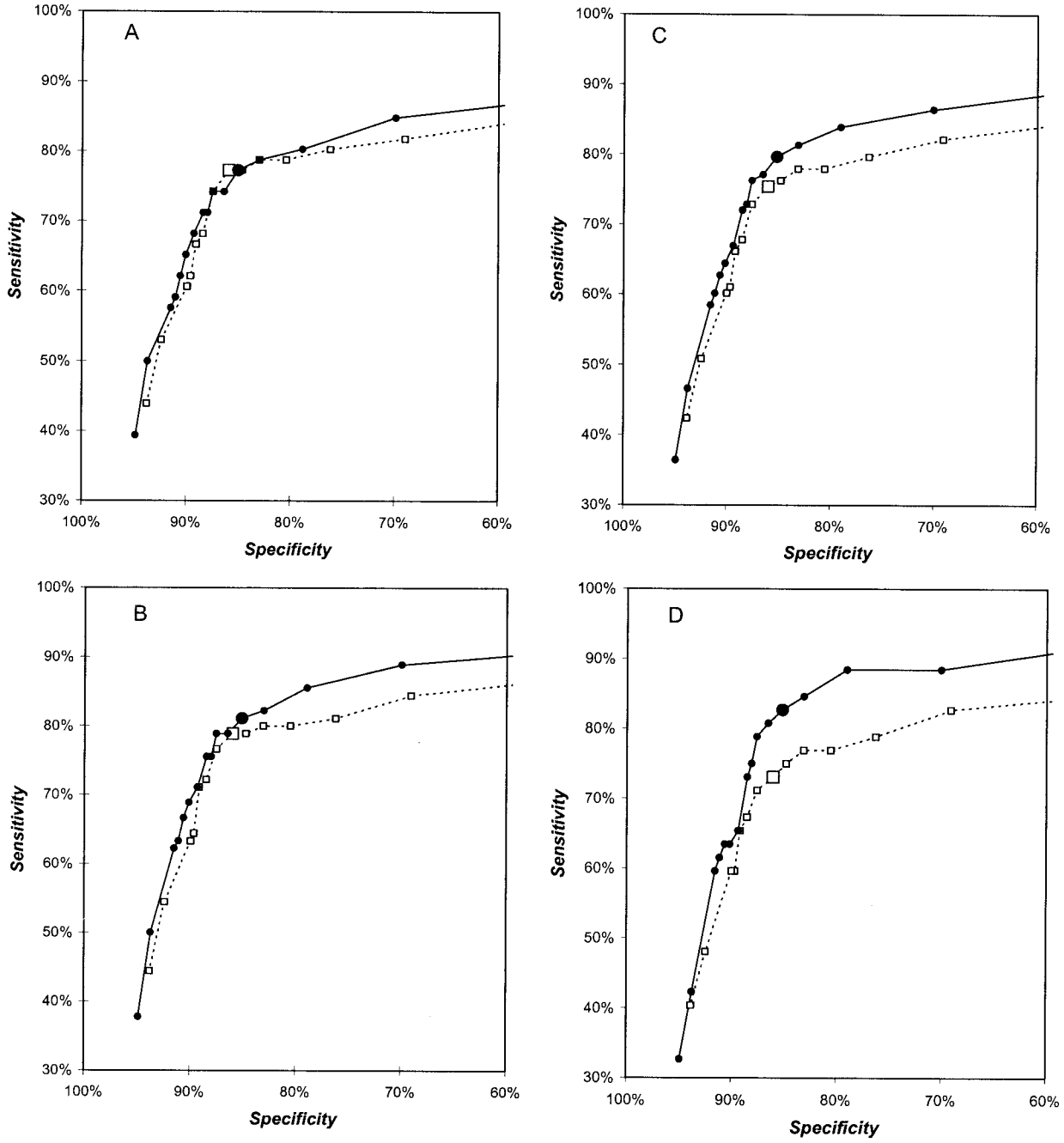

FIG. 1. Receiver operating characteristics (ROCs) for detection of cervical intraepithelial neoplasia grade 3 and cancer (CIN3+) by HPV DNA assays HC2 (■) and HC3 (●). ROCs were calculated for enrollment cases (A), for enrollment cases and those diagnosed within 1 year (B), for enrollment cases and those diagnosed within 3 years (C), and for cases diagnosed within 3 years excluding enrollment cases (D). Enlarged symbols indicate the 1.0-pg/ml cut point for HC2 and the 0.6-pg/ml cut point for HC3.

58, 59, and 68) as targeted by HC2, we tested a risk-stratified sample of 4,364 cervicovaginal lavage specimens collected from women enrolled in a 10-year cohort study at Kaiser Permanente in Portland, Oreg. (11). Specimens were also tested with single HC3 probes specific for HPV16 and HPV18. We compared HC3 test results to previously determined HC2 test results obtained for the entire cohort of 20,810 women (13), assessed the optimal threshold for detection of CIN3+, compared cross-reactivity of both tests with untargeted HPV types with MY09/11 L1 consensus primer PCR results as the referent standard (7), and compared test results of single HPV16- and HPV18-specific HC3 probes to those for PCR.

MATERIALS AND METHODS

Study subjects. Between 1 April 1989 and 2 November 1990, 23,702 women were enrolled in a natural history study of HPV infection at the Kaiser Permanente Northwest Region prepaid health plan in Portland, Oreg., as previously

TABLE 1. Comparison of cut points and corresponding assay performance for HC2 and HC3 for the detection of CIN3+ in all women

CIN3+ group	Test	Cut point (RLU/PC)	No. positive/no. tested	Est. no. positive/no. Tested[a]	Sensitivity[b]	Specificity[b]	Youden's index[b,c]	Referral[b]
Enrollment (n = 66)	HC2	1.0	2,979/20,810		77.3 (67.2–87.4)	85.9 (85.4–86.4)	63.2 (53.0–73.3)	14.3 (13.8–14.8)
	HC3	1.0	1,947/4,345	2,464/20,810	71.2 (60.3–82.1)	88.4 (86.9–89.8)	59.6 (48.5–70.6)	11.8 (10.4–13.3)
	HC3	0.6	2,156/4,345	3,166/20,810	77.3 (67.2–87.4)	85.0 (83.5–86.5)	62.3 (52.1–72.5)	15.2 (13.7–16.7)
0–1 yr (n = 90)	HC2	1.0	2,979/20,810		78.9 (70.5–87.3)	86.0 (85.5–86.4)	64.9 (56.4–73.3)	14.3 (13.8–14.8)
	HC3	1.0	1,947/4,345	2,464/20,810	75.6 (66.7–84.4)	88.4 (87.0–89.9)	64.0 (55.0–73.0)	11.8 (10.4–13.3)
	HC3	0.6	2,156/4,345	3,166/20,810	81.1 (73.0–89.2)	85.1 (83.6–86.6)	66.2 (58.0–74.4)	15.2 (13.7–16.7)
0–3 yr (n = 118)	HC2	1.0	2,979/20,810		75.4 (67.7–83.2)	86.0 (85.6–86.5)	61.5 (53.7–69.2)	14.3 (13.8–14.8)
	HC3	1.0	1,947/4,345	2,464/20,810	72.0 (63.9–80.1)	88.5 (87.0–90.0)	60.5 (52.3–68.8)	11.8 (10.4–13.3)
	HC3	0.6	2,156/4,345	3,166/20,810	79.7 (72.4–86.9)	85.2 (83.7–86.7)	64.8 (57.4–72.3)	15.2 (13.7–16.7)

[a] Estimated number of positive tests for the entire cohort of 20,810 based on the extrapolation of the test performance within each sample of risk strata.
[b] Values are percentages. Values in parentheses are 95% confidence intervals.
[c] Youden's index values are presented as summary statistics of clinical performance.

described (11, 13). Both National Institutes of Health and Kaiser Permanente Institutional review boards approved the study. Subjects were 16 years of age or older, with a mean age of 35.9 years (range, 16 to 94 years). A main analysis cohort of 20,810 women was established and followed passively as part of standard cytologic screening for cervical neoplasia (13). This analysis cohort excluded women who refused to participate (n = 1,107), had undergone hysterectomy (n = 1,406), had an inadequate specimen for HPV testing (n = 195), had unsatisfactory or missing enrollment cervical smears (n = 85), or underwent colposcopy rather than Pap smear screening at enrollment (n = 99).

Enrollment examination. Subjects who consented as required by institutional review boards at Kaiser Permanente and the National Institutes of Health underwent a routine pelvic examination (11). Exfoliated cervical cells were collected with an Ayre spatula and a cytobrush for Pap test screening. Next, a 10-ml sterile saline cervicovaginal lavage was performed on each subject to collect specimens for HPV testing. Lavages were performed by rinsing the cervical os with 10 ml of sterile physiologic saline with a syringe fitted with an intracatheter extender and then collecting the pooled fluid in the vaginal fornix with the same device (11).

Lavages were refrigerated within 1 h of collection and shipped to a central laboratory for processing. A 1-ml aliquot was removed for HPV DNA testing by PCR as described elsewhere (8, 12). The remaining volume was split into two aliquots of approximately equal volume and centrifuged at 400 × g for 5 min (4°C). After drawing off ≈3 ml of supernatant from the top of each aliquot, the pellet and remaining fluid were suspended, repelleted with a microcentrifuge (16,000 × g for 5 min), and the supernatant was decanted. The resulting dry pellets were then frozen at −70°C until used for HPV DNA testing.

Pathology. Tests reported as "normal" or "benign reactive atypia" were reclassified as "negative for intraepithelial lesion or malignancy (negative)" according to the Bethesda 2001 classification (17). Tests reported as "severe reactive atypia, possibly dysplasia" or "possible koilocytotic or condylomatous atypia" were classified as "atypical squamous cells." Cytologic interpretations of dysplasia were reclassified as low- and high-grade squamous intraepithelial lesions.

TABLE 2. Comparison of assay performance for HC2 (1.0 RLU/PC cut point) and HC3 (0.6 RLU/PC cut point) for the detection of CIN3+ in women who were cytologically negative at baseline, women who were aged 30+ years with any cytology, and women who were cytologically negative and aged 30+ years[a]

Stratum	Test	Group (no. of cases)	No. positive/no. tested	Est. no. Positive/no. tested	Sensitivity[a]	Specificity[a]	Youden's index[a,b]	Referral[a]
Cytologically negative	HC2	Enrollment (15)	2,562/20,156		66.7 (42.8–90.5)	87.3 (86.9–87.8)	54.0% (30.1–77.9)	12.7% (12.3–13.2)
	HC3		1,768/3,843	2,772/20,156	66.7 (42.8–90.5)	86.3 (84.7–87.9)	53.0% (29.1–76.9)	13.7% (12.2–15.3)
	HC2	0–1 yr (33)	2,562/20,156		78.8 (64.8–92.7)	87.4 (86.9–87.9)	66.2% (52.2–80.2)	12.7% (12.3–13.2)
	HC3		1,768/3,843	2,772/20,156	84.9 (72.6–97.1)	86.4 (84.8–88.0)	71.2% (58.9–83.6)	13.7% (12.2–15.3)
	HC2	0–3 yr (60)	2,562/20,156		73.3 (62.1–84.5)	87.5 (87.0–87.9)	60.8% (49.6–72.0)	12.7% (12.3–13.2)
	HC3		1,768/3,843	2,772/20,156	80.0 (70.0–90.1)	86.5 (84.9–88.0)	66.5% (56.2–76.7)	13.7% (12.2–15.3)
30+ years old	HC2	Enrollment (36)	1,204/13,419		77.8 (64.2–91.4)	91.2 (90.7–91.7)	69.0% (55.4–82.6)	9.0% (8.5–9.5)
	HC3		725/2,061	1,393/13,419	75.0 (60.9–89.2)	89.8 (87.7–91.9)	64.8% (50.5–79.1)	10.4% (8.3–12.4)
	HC2	0–1 yr (45)	1,204/13,419		75.6 (63.0–88.1)	91.3 (90.8–91.7)	66.8% (54.2–79.4)	9.0% (8.5–9.5)
	HC3		725/2,061	1,393/13,419	77.8 (65.6–89.9)	89.9 (87.8–91.9)	67.6% (55.3–80.0)	10.4% (8.3–12.4)
	HC2	0–3 yr (58)	1,204/13,419		70.7 (59.0–82.4)	91.3 (90.8–91.8)	62.0% (50.3–73.7)	9.0% (8.5–9.5)
	HC3		725/2,061	1,393/13,419	81.0 (71.0–91.1)	89.9 (87.9–92.0)	71.0% (60.7–81.3)	10.4% (8.3–12.4)
30+ years old & cytologically negative	HC2	Enrollment (9)	1,078/13,133		55.6 (23.1–88.0)	91.8 (91.4–92.3)	47.4% (14.9–79.9)	8.2% (7.7–8.7)
	HC3		613/1,882	1,281/13,133	55.6 (23.1–88.0)	90.3 (88.2–92.4)	45.8% (13.3–78.4)	9.8% (7.6–11.9)
	HC2	0–1 yr (16)	1,078/13,133		62.5 (38.8–86.2)	91.9 (91.4–92.3)	54.4% (30.6–78.1)	8.2% (7.7–8.7)
	HC3		613/1,882	1,281/13,133	75.0 (53.8–96.2)	90.3 (88.2–92.5)	65.3% (44.0–86.7)	9.8% (7.6–11.9)
	HC2	0–3 yr (28)	1,078/13,133		60.7 (42.6–78.8)	91.9 (91.4–92.4)	52.6% (34.5–70.7)	8.2% (7.7–8.7)
	HC3		613/1,882	1,281/13,133	82.1 (68.0–96.3)	90.4 (88.3–92.5)	72.5% (58.2–86.9)	9.8% (7.6–11.9)

[a] Values are percentages. Values in parentheses are 95% confidence intervals.
[b] See Table 1, footnote *c*.

TABLE 3. Comparison of MY09/11 L1 consensus PCR, HC3, and HC2 detection of HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 (targeted types); interassay comparisons

Test and result	HC2			HC3		
	No. negative	No. positive	Total	No. negative	No. positive	Total
HC3						
Negative	374	144	518			
Positive	45	684	729			
Total	419	828	1,247			
PCR						
Negative	284	221	505	386	119	505
Positive	135	607	742	132	610	742
Total	419	828	1,247	518	729	1,247

Women with rigorously defined histopathologic CIN3 or cancer (including endocervical adenocarcinoma in situ) were designated as cases. To avoid misclassification of less severe lesions as cases, we restricted our case group to women who had received original histopathologic diagnoses of CIN3 or cancer on two different clinical specimens obtained on different dates (usually a diagnostic punch biopsy and a cone performed for treatment) or who met specific review criteria. The review criteria were an original histopathologic diagnosis of CIN2 reviewed as CIN3 or worse or an original histopathologic diagnosis of CIN3 or worse confirmed as at least CIN2. A single pathologist applying stringent criteria performed the reviews.

Follow-up. During the study period, annual cytologic screening of women at Kaiser Permanente was standard practice. Tests were generally obtained at clinic visits if screening had not been performed within the prior 9 months or there was clinical suspicion of a cervical abnormality. Patients with abnormal cytology were managed according to standard practice guidelines at Kaiser Permanente, which included ablative or excisional treatment for histologic diagnoses of cervical intraepithelial neoplasia grade 2 (CIN2) or worse and some CIN1. HPV testing was not used to direct patient management.

Follow-up time was divided into an initial period of 9 months to capture overt prevalent disease, followed by analogous yearly intervals (9 to 21 months, 21 to 33 months, etc.) to the completion of the study for a total time of 122 months of follow-up. For some analyses (see below), cases that were diagnosed within the first 21 months of follow-up and those that were diagnosed within the first 45 months were included with those that were diagnosed at enrollment. Functionally, it represents the screening that was performed up to the first year and third year of annual screening, respectively.

Hybrid capture HPV testing. Frozen aliquots of cervicovaginal lavage specimens were retrospectively tested. First the entire cohort was tested with HC2 (n = 20,810) (13), and then risk-stratified samples were retested with HC3 (n = 4,364). Both used probe sets that target HPV DNA of 13 cancer-associated HPV types (8). Signal strengths in relative light units (RLU) were compared to 1 pg/ml HPV type 16 DNA-positive controls (PC). Based on a previous study (12), specimens with ≥1 RLU/PC for HC2 testing were a priori considered test positive. Hybrid capture tests were performed at Digene Corporation masked to the clinical results and previous PCR results for HPV DNA testing, and HC3 was also performed masked to the results of the previous HC2 testing.

HC3 was performed on a risk-stratified sample of 4,364 enrollment cervicovaginal lavage aliquots. Specimens were selected and tested based on five hierarchical sampling criteria: (i) to permit direct calculations of assay sensitivity, all 171 cases of CIN3+ diagnosed over the 10-year study (n = 171 results, 100%); (ii) 867 women who were not diagnosed with CIN3+ but who were previously reported to be test positive by MY09/11 L1 consensus primer PCR (n = 855 results, 98.6%); (iii) All 2,260 women who were either not tested or negative by PCR but positive by HC2 (n = 2,253 results, 99.7%); (iv) the 23 women with an enrollment Pap smear interpreted as low squamous intraepithelial lesion or more severe and not captured by the above strata (n = 23 results, 100%). (v) A 6% random sample of the remaining 17,489 women not included in the above strata was tested (n = 1,043 results, 100%). Overall, we had results on 4,345 of 4,364 specimens (99.6%) tested. For type-specific assays with HPV16 and HPV18 probes, there were 4,321 test results (99.0%).

TABLE 4. Interassay comparison statistics[a]

Test	% Exact agreement		Kappa (95% CI)		McNemar's χ^2	
	HC2	HC3	HC2	HC3	HC2	HC3
HC3	84.8		0.68 (0.62–0.73)		<0.001 (HC2)	
PCR	69.1	79.9	0.39 (0.34–0.44)	0.58 (0.53–0.64)	<0.0001 (PCR)	0.4

[a] In the final column, the test with the greatest positivity for oncogenic HPV is shown in parentheses.

MY09/MY11 L1 consensus primer PCR. MY09/MY11 L1 consensus primer PCR (MY09/11 PCR) test results were available from previous studies in this cohort (n = 1,247) (7, 11). A first batch of MY09/11 PCR testing was performed at Cetus Corporation (M. M. Manos), and then a second batch of testing was performed at Albert Einstein College of Medicine (R. D. Burk). Proteinase K-digested cellular specimens were tested for HPV by MY09/MY11 L1 consensus primer PCR with AmpliTaq polymerase (Perkin Elmer Cetus, Norwalk, Conn.) (7). Primers for β-globin were used as the internal PCR amplification control. Amplified DNA was separated by electrophoresis, transferred to a nylon membrane, and hybridized with radiolabeled generic probes for HPV.

Specimens (n = 927) at the M. M. Manos laboratory were tested for the following individual types: 6/11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51 to 59, 66, 68, 73, 82 [W13B], 83 [PAP291], and 84 [PAP155]. Specimens (n = 342) at the R. D. Burk laboratory were tested for the following individual types: 2, 6, 11, 13, 16, 18, 26, 31-5, 39, 40, 42, 45, 51 to 59, 61, 66 to 70, 71 [AE8], 72-4, 81 [AE7], 82 [W13B], 82v [AE2], 83 [PAP291], 84 [PAP155], 85 [AE5], and AE6. Thirty-two specimens were tested by both laboratories, and the results were combined such that positive for any type by either assay was considered positive for that type. The HPV test positive specimens that were negative by all type-specific probes were classified as HPV test positive with undetermined HPV types and were excluded from type-specific analysis. Those specimens demonstrating only weak signals when hybridized with the generic HPV probe and were negative for type-specific probes were conservatively considered to be test negative.

Statistical analysis. To extrapolate HC3 data to the whole cohort, sensitivity percentages were calculated directly (as all high-grade lesions and cancers were tested). To compute test positivity in the whole study population, numbers of HPV-positive specimens for each of the five sampling strata were divided by sampling fractions to derive the number of estimated HPV-positive test results derived from that stratum. Numbers of positive test results expected from each expanded stratum were added to obtain a total estimate of positive test results, which was divided by the number of women in the cohort to obtain the percentage of positive tests in the population. Sensitivity, specificity, and referral rates were all calculated with 95% confidence intervals (95% CI).

Referral rates were based on the assumption that a positive test would be the basis of colposcopic referral (i.e., referral rate equals test positivity). A measurement of test accuracy, Youden's index, was also calculated with 95% CI (Youden's index equals percent sensitivity plus percent specificity − 100%; a test with perfect sensitivity and specificity has a Youden's index of 100%) (14). We evaluated the statistical significance of differences in accuracy (i.e., test positivity for cases and test negativity for noncases) with a newly developed Z-test (Guillermo Marshall, in preparation) to account for screening accuracy for the two tests being evaluated in the same individuals and to give equal weight to sensitivity and specificity, just as Youden's index does. The Z statistic is computed according to the formula:

$$Z = \frac{\frac{n_{+-} - n_{-+}}{n} - \frac{m_{+-} - m_{-+}}{m}}{\sqrt{\frac{n_{-+} + n_{+-} - [n_{+-} - n_{-+}]^2/n}{n^2} + \frac{m_{-+} + m_{+-} - [m_{+-} - m_{-+}]^2/m}{m^2}}}$$

where n is the number of cases, m is the number of noncases, n_{+-} is the number of cases classified properly as positive only by test strategy 1, n_{-+} is the number of cases classified as positive by test strategy 2 only, m_{-+} is the number of noncases classified properly as negative by test strategy 1 only, and m_{+-} is the number of noncases classified properly as negative by test strategy 2 only. Only discordant test results figure into this statistic, and women with missing values for either test were excluded.

Receiver operating characteristic (ROC) analyses (20), modified by using specificity instead of 1 − specificity, for detection of CIN3+ diagnosed at enrollment currently with cervicovaginal lavage specimen collection (n = 66) were performed for both HC2 and HC3 with the following cut points: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 5.0, and 10.0 RLU/PC (there is a linear relationship between RLU/PC and picogram per milliliter values above 1

TABLE 5. Association of CIN3+ (odds ratios [OR] and 95% CI) with the HPV types listed for Tables 3 and 4 as detected by the three methods

Group	Total no.	MY09/11 PCR		HC3		HC2	
		No. positive	OR (95% CI)	No. positive	OR (95% CI)	No. positive	OR (95% CI)
Enrollment							
Controls	1,195	699	1	686	1	785	1
Cases (CIN3+)	52	43	3.89 (1.64–7.02)	43	3.55 (1.71–7.34)	43	2.50 (1.20–5.17)
0–1 yr							
Controls	1,175	682	1	688	1	768	1
Cases (CIN3+)	72	60	3.61 (1.92–6.79)	61	4.21 (2.19–8.08)	60	2.65 (1.41–4.98)
0–3 yr							
Controls	1,155	671	1	655	1	756	1
Cases (CIN3+)	92	71	2.44 (1.48–4.02)	74	3.14 (1.85–5.32)	72	1.90 (1.14–3.16)

RLU/PC for HC2) (8, 17). The cut point of 1.0 RLU/PC (= 1.0 pg/ml) is standard for the HC2 (12); we wished to confirm this choice with cervicovaginal lavage specimens. To address the possibility that Pap screening at enrollment missed some prevalent cases or that some cases developed rapidly, separate ROC analyses were performed to include those cases diagnosed at enrollment through the first year of follow-up (0- to 1-year cases) ($n = 90$), those cases diagnosed at enrollment through the first 3 years of follow-up (0- to 3-year cases) ($n = 118$), and those cases diagnosed in the first 3 years of follow-up excluding the enrollment cases ($n = 52$). With the optimal cut points from these ROC analyses, we also examined the performance characteristics of these tests on groups of women that might be targeted by HPV screening: women 30 years and over, cytologically negative women, and the combination. Additional analyses that included unreviewed histologic CIN2 cases ($n = 111$, enrollment; $n = 149$, 0- to 1-year cases; $n = 199$, 0- to 3-year cases) were also performed.

For the subset of specimens for which MY09/11 PCR, HC2, and HC3 data were available ($n = 1,247$), we also evaluated HPV type specificity of the two hybrid capture tests by comparing the test results to referent PCR test results with contingency tables and Pearson χ^2 tests. MY09/11 PCR data were ranked hierarchically: (i) test positive for the 13 cancer-associated (high-risk) HPV types targeted by hybrid capture assays, else (ii) test negative for the targeted HPV types but positive for one or more untargeted HPV types (including HPV DNA positives by the generic probe but uncharacterized by type-specific probes), else (iii) HPV DNA negative. HC2 and HC3 data were categorized by whether both tests were negative (HC2−/HC3), HC2 was negative but HC3 was positive (HC2−/HC3+), HC2 was positive but HC3 was negative (HC2+/HC3−), or both tests were positive (HC2+/HC3+). Test results for 13 targeted high-risk types by all three HPV DNA assays were compared with kappa analyses and McNemar χ^2.

To examine the test positivity for single targeted types and the predilection for test positivity for untargeted types (cross-reactivity), HC3 test results were compared to HC2 test results with McNemar's χ^2 restricted to PCR test positives for a single HPV type. As a crude measure for comparing the sensitivity and specificity of each assay among the women with valid tests for all three assays, logistic regression was used to calculate the crude odds ratios (OR) and 95% CI as an estimate of the association of these HPV types with CIN3+.

PCR data were also used to evaluate the test results of single type-specific HC3 probes for HPV16 and HPV18 by with kappa analyses and McNemar χ^2 tests. Logistic regression, restricted to the 1,189 women with test results for the type 16 and 18 probes and for PCR, was again used to calculate the OR and 95% CI to estimate the association of hierarchical HPV categories (HPV16 > HPV18 > other targeted high-risk types > all other types and HPV DNA negatives), as detected by PCR and HC3, with CIN3+.

RESULTS

An ROC analysis was performed to determine the optimal cut point for HC3, based on testing a risk-stratified sample of the cohort and extrapolating those results to the cohort. A parallel analysis for HC2, based on previous testing of the entire cohort (13), was performed because cervicovaginal lavage samples are not the standard cervical specimen for HPV DNA detection by hybrid capture and the effect of using this type of specimen on assay performance is unknown.

As shown in Fig. 1A to C, the ROC curves for both HC3 and HC3 with and without early follow-up cases have inflection points at ≈75% to 80% sensitivity and 85% to 90% specificity. At specificities lower than the inflection point, HC3 was more sensitive than HC2 for a given specificity, and this difference was greater when including early follow-up cases. Figure 1D shows the ROC curves for cases that were diagnosed after enrollment but within 3 years, showing that the primary analytic distinction between HC3 and HC2 appeared to be the ability of HC3 to detect these early follow-up cases. The optimal cut point for HC3 and HC2 was 0.6 RLU/PC and 1.0 RLU/PC, respectively, for enrollment cases, 0- to 1-year cases, and 0- to 3-year cases.

TABLE 6. Comparison of HC2 and HC3 HPV type specificity with MY09 L1 consensus primer PCR results available for 1,247 specimens[a]

HPV group (PCR)	No. (%) of cases				
	HC2−/HC3−	HC2−/HC3+	HC2+/HC3−	HC2+/HC3+	Total
Negative	139 (53.6)	8 (3.1)	68 (26.1)	46 (18.5)	261 (100.0)
Untargeted types	132 (51.6)	5 (2.1)	62 (25.4)	45 (18.4)	244 (100.0)
Targeted types	118 (15.9)	17 (2.3)	29 (3.9)	578 (77.9)	742 (100.0)
Total	389	30	159	669	1,247

[a] PCR data were classified hierchically: targeted types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68), if not target then untargeted types, else negative. HC2 (1.0-pg/ml cut point) and HC3 (0.6-pg/ml cut point) data were categorized by whether both tests were negative (HC2−/HC3−), HC2 was negative but HC3 was positive (HC2−/HC3+), HC2 was positive but HC3 was negative (HC2+/HC3−), or both tests were positive (HC2+/HC3+). Raw percentages are shown in italics. $P < 0.001$, Pearson χ^2, overall; $P < 0.001$, Pearson χ^2; HC2−/HC3+ versus HC2+/HC3−.

TABLE 7. Comparison of type-specific detection of HPV16 by an HC3 HPV16 probe (0.6 pg/ml) to detection by MY09/11 L1 consensus primer PCR for 1,189 women with valid PCR results and type-specific HC3 data[a]

PCR result	No. of women with HC3 HPV16 result that was[b]:		Total
	Negative	Positive	
HPV16 negative	918 (97.6)	23 (2.4)	941 (100.0)
HPV16 positive	64 (25.8)	184 (74.2)	248 (100.0)
Total	982	207	1,189

[a] Exact agreement = 92.6%, κ = 0.76 (95% CI = 0.71–0.82), McNemar's $\chi^2 < 0.001$.
[b] Values in parentheses are raw percentages.

TABLE 8. Comparison of type-specific detection of HPV18 by an HC3 HPV18 probe (0.6 pg/ml) to detection by MY09/11 L1 consensus primer PCR for 1,189 women with valid PCR results and HC3 data[a]

PCR result	No. of women with HC3 HPV18 result that was[b]:		Total
	Negative	Positive	
HPV18 negative	1.091 (98.8)	13 (1.2)	1,104 (100.0)
HPV18 positive	26 (30.6)	59 (69.4)	85 (100.0)
Total	1,232	75	1,189

[a] Exact agreement = 96.8%, κ = 0.73 (95% CI = 0.68–0.79), McNemar's $\chi^2 = 0.04$.
[b] Values in parentheses are raw percentages.

The assay performances of these tests are summarized in Table 1. The referral rate (equal to the percentage of women with at least one of the 13 targeted HPV types) for HC3 was estimated to be 15.2% (95% CI = 13.7% to 16.7%), compared to a referral rate of 14.3% (95% CI = 13.8% to 14.8%) for HC2. For enrollment cases alone, the accuracy of HC3 positivity (≥0.6 RLU/PC), as measured by Youden's index, was similar to HC2 positivity (≥1.0 RLU/PC) (Table 1). HC3 positivity (≥0.6 RLU/PC) was nonsignificantly more sensitive and had similar specificity compared to HC2 positivity (≥1.0 RLU/PC) when early cases of CIN3+ were included in the analysis. Inclusion of unreviewed CIN2 diagnoses into our case definition did not appreciably alter the performances of these two tests (data not shown).

We examined the performances of HC3 positivity (≥0.6 RLU/PC) (extrapolated results based on testing a risk-stratified sample) and HC2 positivity (≥1.0 RLU/PC) in groups of women that might be targeted for screening and ancillary testing for negative reassurance: in women of all ages who were cytologically negative at enrollment, women who were aged 30+ years regardless of cytology result, and women who were cytologically negative and aged 30+ years (Table 2). In these subgroups, there was no difference in performance (as measured by Youden's index) of HC3 positivity (≥0.6 RLU/PC) and HC2 positivity (≥1.0 RLU/PC) for detection of enrollment cases. For 0- to 3-year cases, HC3 positivity (≥0.6 RLU/PC) was nonsignificantly more accurate than HC2 positivity (≥1.0 RLU/PC) among cytologically negative women and among women who were 30+ years of age. Among the cytologically negative women aged 30+ years, the improved accuracy of HC3 positivity (≥0.6 RLU/PC) compared to HC2 positivity (≥1.0 RLU/PC) was significant ($P = 0.006$).

The exact agreement and kappa value for HC3 positivity (≥0.6 RLU/PC) and HC2 positivity (≥1.0 RLU/PC) were 76.6% and 0.53 (95% CI = 0.51 to 0.56), respectively, with specimens more likely to be HC2 positive (≥1.0 RLU/PC) for the 4,345 specimens tested by both assays ($P < 0.0001$, McNemar's χ^2). A subset of 1,247 specimens also had MY09/11 PCR test results, and we examined the interassay agreement of the three HPV DNA assays for the 13 HPV types targeted by hybrid capture assays (Tables 3 and 4). Overall, in this subset of specimens selected for high HPV DNA prevalence, there was 66.4% HC2 positivity (≥1.0 RLU/PC), 58.5% HC3 positivity (≥0.6 RLU/PC), and 59.5% PCR positivity for the targeted types (all type PCR test positivity was 79.1%). The exact agreement and kappa value for HC3 positivity (≥0.6 RLU/PC) versus HC2 positivity (≥1.0 RLU/PC) were 84.8% and 0.68 (95% CI = 0.62 to 0.73), respectively, with specimens more likely to be test positive by HC2 ($P < 0.0001$, McNemar's χ^2). For HC2 positivity (≥1.0 RLU/PC) versus PCR positivity, the exact agreement and kappa value were 69.1% and 0.39 (95% CI = 0.34 to 0.44), respectively, with specimens more likely to be test positive by HC2 ($P < 0.0001$, McNemar's χ^2). For HC3 positivity (≥0.6 RLU/PC) versus PCR positivity, the exact agreement and kappa value were 79.0% and 0.58 (95% CI = 0.53 to 0.64), respectively, and there was no difference in the positivity between the two tests ($P = 0.4$, McNemar's χ^2). The OR values for the association of test positivity with CIN3+, as detected by each method, indicated that each method tested positive for a similar proportion of HPV in cases compared to HPV noncases, although HC2 detection had consistently but nonsignificantly lower OR than detection by other methods (Table 5).

We next compared the paired test results of HC3 (≥0.6 RLU/PC) and HC2 (≥1.0 RLU/PC) to the referent MY09/MY11 PCR test results categorized as negative, untargeted types, and targeted types to evaluate the discordant test results (HC2+/HC3− and HC2−/HC3+). As shown in Table 6 and Appendix 1 (Tables A1 and A2), a greater proportion of specimens classified by PCR as either negative specimens or specimens with untargeted types were HC2+/HC3− than were HC2−/HC3+ ($P < 0.0001$, Pearson χ^2). HC3 (≥0.6 RLU/PC) was slightly more likely than HC2 (≥1.0 RLU/PC) to test positive for specimens that tested positive by PCR for single type infections HPV18 ($P = 0.08$, McNemar's χ^2), HPV45 ($P = 0.08$, McNemar's χ^2), HPV51 ($P = 0.08$, McNemar's χ^2), and HC2 was marginally more likely than HC3 to test positive for specimens that tested positive by PCR for single type infections HPV56 ($P = 0.1$, McNemar's χ^2). There were no differences between HC2 positivity (≥1.0 RLU/PC) and HC3 positivity (≥0.6 RLU/PC) for specimens that tested positive by PCR for HPV16 ($P = 0.7$). Restricting to single PCR-positive types not targeted by hybrid capture assays, HC2 was more likely than HC3 to test positive for HPV53 ($P = 0.001$, McNemar's χ^2) and HPV66 ($P = 0.01$, McNemar's χ^2) (Table A2).

We also compared the test positivity by individual HC3

TABLE 9. Comparison of odds ratio and 95% CI for the association of hierarchical HPV risk groups (HPV16 > HPV18 > other targeted high-risk HPV types[a] > other HPV types and HPV negative) with CIN3+

Cases	MY09/11 PCR			HC3		
	No. of controls	No. of cases	OR[b] (95% CI)	No. of controls	No. of cases	OR (95% CI)
Baseline						
Untargeted HPV types & PCR negative	496	9	1	499	9	1
Other targeted types	414	9	1.20 (0.471–3.05)	454	13	1.59 (0.672–3.75)
HPV18	61	3	2.67 (0.703–10.1)	58	2	1.91 (0.403–9.06)
HPV16	211	31	7.66 (3.59–16.4)	184	28	8.44 (3.91–18.2)
0–1 yr						
Untargeted HPV types & PCR negative	493	12	1	497	11	1
Other targeted types	410	13	1.80 (0.588–2.89)	448	19	1.92 (0.902–4.07)
HPV18	61	4	2.69 (0.842–8.61)	59	2	1.56 (0.337–7.20)
HPV16	211	49	8.37 (4.33–16.2)	172	40	10.5 (5.27–20.9)
0–3 yr						
Untargeted HPV types & PCR negative	484	21	1	492	16	1
Other targeted types	405	18	1.02 (0.538–1.95)	439	28	1.96 (1.05–3.67)
HPV18	61	4	1.51 (0.502–4.55)	57	3	1.62 (0.458–5.72)
HPV16	205	49	5.51 (3.22–9.42)	167	45	8.29 (4.56–15.1)

[a] HPV types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68.
[b] OR, odds ratio.

probes (≥0.6 RLU/PC) for HPV16 and HPV18 to that of PCR positivity for these types (Table 7). For HPV16, there was an exact agreement of 92.6% and a kappa value of 0.75 (95% CI = 0.69 to 0.80), with specimens more likely to be test positive by PCR ($P < 0.001$, McNemar's χ^2) (Table 7). For HPV18, there was an exact agreement of 96.8% and a kappa value of 0.72 (95% CI = 0.67 to 0.77), with specimens marginally more likely to be test positive by PCR ($P = 0.04$, McNemar's χ^2) (Table 8). Finally, there was a nonsignificant but consistent lesser tendency for HC3 HPV16 positivity (≥0.6 RLU/PC) in controls than PCR detection as reflected in the slightly higher OR values shown in Table 9.

DISCUSSION

We present the first data on the performance of a prototype version of HC3, possibly the next generation of hybrid capture test for targeting the 13 HPV types most strongly associated with cervical cancer. Based on these analyses, the optimal cut point for HC3, 0.6 RLU/PC, was lower than the optimal cut point of the HC2 test, 1.0 RLU/PC. HC3 at this cut point had very similar performance to HC2 for detection of enrollment cases. However, HC3 was nonsignificantly more sensitive than and equally specific as HC2 for detection of CIN3+ when both enrollment and early follow-up cases were included in the analysis, particularly in women who were 30 years and older and were cytologically negative by conventional Pap, perhaps as the result of using a lower threshold for a positive test. Since the interval for diagnosing of CIN3 from the initial HPV infection averages 5 years (7) or more, the inclusion of CIN3+ during follow-up of 1 or 3 years most likely represents missed detection by standard Pap screening but could also represent enrollment HPV infection that has not yet (rapidly) progressed to recognizable CIN3+.

HC3 was designed to minimize cross-reactivity with untargeted, generally low-risk HPV types that HC2 occasionally detects. We examined the fidelity of these two tests by comparing test results to PCR test results, our referent standard, in a subset of specimens with very high HPV "prevalence," a choice which would emphasize the phenomenon of cross-reactivity. The overall test positivity for targeted types was higher for HC2 than for HC3 or PCR (Table 3), but comparison of discordant test results (HC2 versus HC3) (Table 6) reveals that many of the additional test positives for HC2 were classified as untargeted (nononcogenic) types. Thus, discrepancies in sensitivity between HC2 and the other assays for the targeted types appear to be the result of cross-reactivity (false positivity) with untargeted types rather than true greater sensitivity by HC2. We also note that the overall agreement test positivity between PCR and HC3 (0.6 RLU/PC) for targeted types was better than the agreement between PCR and HC2 (1.0 RLU/PC), suggesting that HC3 was performing more like PCR in terms of type fidelity.

Systematic studies comparing HC2 testing to type-specific PCR data demonstrated test positivity for untargeted HPV types 53, 66, 67, and 71 as well as occasional test positivity to other untargeted HPV types (3, 11, 19); the impact of the test positivity for untargeted HPV types on screening performance will depend on the prevalence of these types in different populations. In our analyses, we found that the test positivity for untargeted HPV types 53 and 66 was significantly less with HC3 (0.6 RLU/PC) than with HC2 (1.0 RLU/PC). No infections of type 67 and one infection of type 71 were identified by PCR in this set of specimens, and thus HC2 and HC3 could not be evaluated for these types. It is of note that there is now evidence to suggest that type 66 and a few other questionably high-risk HPV types confer appreciable risk of cervical cancer (9), and therefore the inclusion of additional probes for these types may theoretically improve the performance of subsequent versions of HC3. Since test positivity in HC3 can be

tightly controlled by the design of the capture oligonucleotides and the temperature of the hybridization reaction, future work may investigate if the reintroduction of a small degree of reactivity for these types may benefit the clinical performance of the test.

One limitation of this study is the use of cervicovaginal lavage specimens, which are neither the standard nor optimal specimens for the hybrid capture tests and not the standard collection as part of regular cytology screening. To overcome this limitation, we included parallel ROC analyses of HC2 for the purpose of comparison. Although the overall performance of HC2 testing was lower than what has been observed in other studies of HC2 (9), we note that the optimal cut point for HC2 was again 1.0 RLU/PC, as had been observed with directly sampled cervical specimens (13), suggesting that conclusions about the relative performance of HC3 (≥0.6 RLU/PC) are valid. However, we cannot rule out that the use of cervicovaginal lavage samples may have differentially affected the performance of the assays. A second consideration may be that the specimens used are >10 years old, and it is possible that there was some specimen degradation over time. Given the aforementioned differences in the performance of HC2 (≥1.0 RLU/PC) likely attributable to the method of cervical sampling, we suggest that the performance of HC3 (≥0.6 RLU/PC) may be enhanced with the use of fresh, swab- or brush-collected specimens for testing, but this awaits confirmation.

The reasons for the increased positivity of HC3 (≥0.6 RLU/PC) compared to HC2 (≥1.0 RLU/PC) associated with CIN3+ during follow-up are unclear. Given the small number of cases detected by HC3 and not by HC2 during the early follow-up of all women ($n = 5$), we cannot rule out a chance finding. Alternatively, the lower cut point for HC3 compared to HC2 may permit the detection of some lower viral load HPV infections associated with either missed prevalent CIN3+ or CIN3+ that developed rapidly into overt disease during the 3-year follow-up. This optimal lower threshold for positivity for the HC3 assay may reflect its greater ability to distinguish between targeted hybrids and competing nonspecific hybrids. It is noteworthy that HC3 was a more sensitive and equally specific test for CIN3+ compared to HC2 among women who were cytologically negative, a cytologic interpretation that is consistent with lower viral loads (13).

As a proof of principle, we demonstrated typing with HPV16 and HPV18 with single HC3 probes with a cut point of 0.6 RLU/PC. The test positivity of HPV16 and HPV18 probes in HC3 was consistent with detection of these types by PCR, but in contrast to detection of the 13 types with the HC3 probe set, single-type detection by HC3 had a somewhat lower analytical sensitivity than PCR.

For targeted screening of women in some geographic regions, it may be worth considering a higher cut point for HC3 (or HC2) to reduce referral rates as the result of increased specificity while maintaining high sensitivity. For example, HC3 at a cut point of 0.8 RLU/PC would result in sensitivities, specificities, and Youden's indices of 74.2%, 87.4%, and 61.7%, respectively, for enrollment CIN3+ and 76.3%, 87.6% and 63.9%, respectively, for 0- to 3-year CIN3+, with a referral rate of 12.8%. Such trade-offs may be worth examining in a formal cost-to-benefit analysis.

In conclusion, we suggest that HC3 (≥0.6 RLU/PC) may be a slightly more sensitive, equally specific test for the detection of CIN3+ over the duration of typical screening intervals compared to its predecessor, HC2 (≥1.0 RLU/PC). The increased sensitivity of HC3 (≥0.6 RLU/PC) compared to HC2 (≥1.0 RLU/PC) appears to be the result of increased detection of CIN3+ in women who were 30 years of age or older and were cytologically negative. We emphasize that further validation studies of HC3 are needed with more clinically relevant cervical specimens.

APPENDIX

Tables A1 and A2 present HC2 and HC3 positivity and cross-reactivity identified by MY09/11 PCR.

TABLE A1. HC2 and HC3 positivity for any HPV infection (single or multiple type) as identified by MY09/11 PCR[a]

Infection	HPV type	No. of tests with result:			
		HC2−/ HC3−	HC2−/ HC3+	HC2+/ HC3−	HC2+/ HC3+
Targeted types	16	44	2	7	201
	18	13	2	1	69
	31	9	2	4	75
	33	5	0	1	38
	35	5	4	1	40
	39	5	1	3	53
	45	11	3	0	43
	51	13	2	1	74
	52	9	3	2	61
	56	4	1	7	72
	58	4	0	3	52
	59	1	1	1	36
	68	4	0	5	17
Untargeted types	2	0	0	0	0
	6	7	0	3	3
	11	5	0	0	0
	13	0	0	0	0
	26	0	0	0	1
	32	0	0	0	0
	40	5	0	1	0
	42	5	0	1	0
	53	29	3	21	1
	54	18	1	3	1
	55	11	0	0	0
	61	10	0	0	1
	66	2	0	7	2
	67	0	0	0	0
	69	0	0	0	0
	70	0	0	1	0
	71	1	0	0	0
	72	1	0	0	0
	73	10	0	1	3
	74	0	0	0	0
	81	2	0	0	1
	82	1	1	2	2
	83	18	0	1	0
	84	13	0	4	3
	85	0	0	0	0
	82v	0	0	1	1
	AE6	0	0	2	1

[a] For untargeted (nononcogenic) HPV type infections, multitype infections that included targeted (oncogenic) types were excluded. Underlining indicates that only one laboratory (R.D.B.) tested for these types. Boldfacing indicate a significant difference (McNemar's χ^2 test) in detection between the two tests.

TABLE A2. Cross-reactivity of HC2 and HC3 with single nononcogenic HPV infections as identified by MY09/11 PCR[a]

HPV type	No. of tests with result:					
	HC2+	HC3+	HC2−/ HC3−	HC2−/ HC3+	HC2+/ HC3−	HC2+/ HC3+
2	0	0	0	0	0	0
6	4	2	3	0	2	2
11	0	0	4	0	0	0
13	0	0	0	0	0	0
26	0	0	0	0	0	0
32	0	0	0	0	0	0
40	0	0	3	0	0	0
42	1	0	4	0	1	0
53	20	4	22	3	19	1
54	3	1	12	1	3	0
55	0	0	9	0	0	0
61	0	0	10	0	0	0
66	7	1	2	0	6	1
67	0	0	0	0	0	0
69	0	0	0	0	0	0
70	1	0	0	0	1	0
71	0	0	1	0	0	0
72	0	0	1	0	0	0
73	3	3	7	0	0	3
74	0	0	0	0	0	0
81	0	0	1	0	0	0
82	3	2	0	1	2	1
83	1	0	14	0	1	0
84	3	2	9	0	1	2
85	0	0	0	0	0	0
82v	2	1	0	0	1	1
AE6	2	0	0	0	2	0

[a] See Table A1, footnote *a* for further details.

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